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Inactivation of Lipid-Containing Viruses by Long-Chain Alcohols

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This report describes the inactivation of lipid-containing viruses by several long-chain alcohols. A striking peak in antiviral activity was found for saturated alcohols having chain lengths from 10 to 14 carbons. Viruses having different membrane structure showed different susceptibilities to alcohols having different chain lengths and structural features. Decanol, dodecanol, and tetradecanol readily inactivated herpes simplex virus and the enveloped bacterial virus ϕ6. The lipid-containing virus PM2 was susceptible to decanol and dodecanol but comparatively unsusceptible to tetradecanol. The branched-chain alcohol phytol, a naturally occurring component of chlorophyll, was active against ϕ6 and herpes simplex virus but not against PM2. Polyoma virus and the bacteriophage ϕ23-1-a, which do not contain lipids, were not susceptible to inactivation by any of the alcohols tested. Experiments were also carried out to determine the effects of these compounds on cells. At 0.5 mM, decanol lysed human embryonic lung cells, erythrocytes, and the bacterial hosts for ϕ6 and PM2. Dodecanol, tetradecanol, and phytol at this concentration were less damaging to cells. At 0.05 mM, none of the alcohols caused observable cytopathic effects on human embryonic lung cells, although several of the alcohols at this concentration were active against herpes simplex virus. Our findings suggest that dodecanol, tetradecanol, and phytol may warrant further studies as potential antiviral agents, particularly for topical application to virus-infected areas of the skin.

Most antiviral agents being developed for clinical use are designed to interfere with viral nucleic acid metabolism. Many of these are structural analogues to deoxyribonucleic acid and ribonucleic acid precursors, their activity being due to their competitive inhibition of enzymatic reactions (2, 8, 10, 13, 16). There are several properties of this class of compounds that limit their desirability for use as antiviral agents. Some of the more serious ones are: (i) their frequently limited selectivity for inhibiting viral nucleic acid synthesis in comparison to that of the cell; (ii) the development of virus mutants, either spontaneous or induced, that are resistant to the agent; and (iii) the potential mutagenic and teratogenic effects that may occur when dividing cells are exposed to nucleoside analogues, particularly those that become incorporated into nucleic acid polymers. These considerations suggest that additional, alternative approaches to the design of antiviral compounds is a worthy endeavor.

The effective treatment and eventual control of many viral diseases will quite likely rely on the development of drugs that complement each other in their activity, acting at different points in the virus life cycle. In our laboratory we are exploring the possibility that certain viruses, namely, those that are enveloped, can be inactivated by hydrophobic, membrane-perturbing molecules under conditions comparatively harmless to the host cells. This approach was prompted by our discovery that butylated hydroxytoluene (BHT), a commonly used food additive with apparent low toxicity to humans, is nevertheless a potent inactivator of lipid-containing viruses, including herpes simplex virus (HSV) (3, 15, 18). This combination of low toxicity and effectiveness against viruses has generated considerable interest in the potential use of BHT as an antiviral agent, particularly for topical application to virus-infectd areas of the skin. Clinical evaluation of the effectiveness of BHT as an antiviral substance is forthcoming.

The proper design of antiviral agents whose action is due to their perturbation of viral membranes will rely on an understanding of their physical properties and the nature of their interactions with membrane structures. We are currently carrying out experiments to investigate the structural parameters important to the

activity of these agents. Compounds that appear to be particularly effective have a hydrophobic component, such as a hydrocarbon chain, to pull the compound into the virus membrane and a polar group to orient the molecule at the membrane-aqueous interface. In the work described in this report, we have investigated the antiviral properties of a series of alcohols varying in chain length from 4 to 18 carbons and the branched-chain alcohol phytol, a naturally occurring component of chlorophyll. A striking peak in activity was observed for alcohols having chain lengths from 10 to 14 carbons, suggesting that a proper balance between the polar and hydrophobic components can lead to maximal perturbation of the viral membrane. For comparative purposes, studies were also carried out to determine the effects of these same compounds on several types of cells, including the host cells in which the virus replicates and erythrocytes. Our findings suggest that some of these compounds, particularly dodecanol, tetradecanol, and phytol, may warrant further studies as potential antiviral agents.

MATERIALS AND METHODS

Viruses and cells. The enveloped bacterial virus ϕ6 was originally isolated by Vidaver et al. (17). The virion contains 25% phospholipid, located in a loose membrane structure similar to that of enveloped animal viruses (17). ϕ6 infects the bacterium *Pseudomonas phaseolicola* strain HB10Y. Our procedures for the routine culture of ϕ6 and HB10Y have been reported (12, 18).

The bacteriophage ϕ23-1-a was obtained from Anne Vidaver. It is insensitive to treatment with organic solvents, such as chloroform and ether, and is presumed to contain no lipid. ϕ23-1-a infects the same host cell as does ϕ6 and is useful in comparative studies for susceptibility to antiviral agents.

The lipid-containing bacterial virus PM2 was isolated by Espejo and Canelo (4). Its structure has been studied extensively by Franklin and co-workers (1, 5, 6). The virion, which contains 13% lipid, has an icosahedral protein coat external to the lipid bilayer (6) and is therefore unlike any known animal viruses. PM2 infects the marine bacterium *Pseudomonas* BAL-31. Procedures for maintaining PM2 and BAL-31 have been described (3, 14).

A syncytia-forming mutant of HSV type 1, designated here HSV-*syn* 20, was used in these experiments. Its isolation and partial characterization have been reported (9). HSV-*syn* 20 was assayed for plaque-forming units on monolayers of human embryonic lung (HEL) cells in plastic petri dishes. The procedures for growth and maintenance of stocks of HSV-*syn* 20 and HEL cells have been published (9).

A wild-type, large-plaque strain of polyoma virus, kindly provided by W. Eckhard was used. The virus was maintained and assayed for plaque-forming units on the mouse embryo cultured cell line 3T3 (A31 clone) obtained from Todaro.

Treatment of virus with alcohols. The longer-chain alcohols having 10 carbons and greater are extremely insoluble in aqueous media. Therefore, the following prcoedure was adopted for treating viruses with these compounds. Stocks of the compounds were prepared in 95% ethanol at 100 times the desired final concentration. A 0.1-ml portion of this stock was added to 5 ml of an appropriate buffered solution, the solution was vortexed, and 30 s later an equal volume of a virus suspension in the same buffered solution was added. After incubation at room temperature for an appropriate time, usually 30 min, the samples were diluted and assayed for plaque-forming units. Minor modifications in volumes, maintaining the same proportions, were used for those viruses available in more limited quantity. None of the viruses are affected by the 1% ethanol present in the culture at the time of treatment. For the longer-chain alcohols at high concentrations, cloudy suspensions formed when the ethanol stocks were added to the aqueous solutions. The concentrations reported, therefore, are those used to form the solution or suspensions and in some cases do not necessarily represent the dissolved concentration of the compound.

Buffer solutions. ϕ6 and ϕ23 were treated in a tris(hydroxymethyl)aminomethane(Tris)-buffered solution containing 6 g of NaCl, 3 g of KCl, 2 g of NH_4Cl, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of KH_2PO_4, and 12.1 g of Tris base per liter of distilled water. The pH was adjusted to 7.6 with concentrated HCl. PM2 was treated in a Tris-buffered solution containing 26 g of NaCl, 9.9 g of $MgCl_2 \cdot 6H_2O$, 1.5 g of $CaCl_2 \cdot 2H_2O$, 1.1 g of NH_4Cl, 0.7 g of KCl, 0.025 g of KH_2PO_4, 0.023 g of Na_2SO_4, and 12.1 g of Tris base per liter of distilled water. The pH was adjusted to 7.6 with concentrated HCl. HSV-*syn* 20 was treated in a tricine-buffered solution containing 8 g of NaCl, 0.38 g of KCl, 0.1 g of $MgCl_2 \cdot 6H_2O$, 0.1 g of $CaCl_2 \cdot 2H_2O$, and 4.5 g of tricine per liter of distilled water. The pH was adjusted to 7.3. Polyoma was treated in a phosphate-buffered solution containing 8 g of NaCl, 0.2 g of KCl, 1.15 g of Na_2HPO_4, 0.2 g of KH_2PO_4, 0.133 g of $CaCl_2 \cdot 2H_2O$, and 0.1 g of $MgCl_2 \cdot 6H_2O$ per liter of distilled water. The pH of this solution was 7.2.

RESULTS

Effects of straight-chain alcohols on ϕ6 and ϕ23-1-a. We have found ϕ6 and ϕ23-1-a to be very convenient for initial studies on the antiviral activity of compounds whose mode of action is through membrane perturbations. ϕ6 is especially susceptible to these compounds, and in most cases those agents effective against ϕ6 are also active, usually at higher concentrations, against HSV. ϕ23-1-a, which infects the same host cell as does ϕ6, is generally unsusceptible to compounds that act on membranes and therefore provides a convenient distinction between the classes of compounds in which we are interested and those that may act by some other mechanism.

Extensive data were accumulated on the effects of straight-chain alcohols on ϕ6 at varying

concentrations. Striking differences were observed in the effectiveness of alcohols with different chain lengths. Figure 1 shows the percentage of inactivation of ϕ6 by alcohols having 8- and 14-carbon chains as a function of concentration of alcohol. The virus was exposed to the compounds for 30 min at room temperature. We have repeatedly observed that the maximum inactivation is reached in less than 15 min, so a 30-min exposure was used for all the experiments reported here. Graphs such as those of Fig. 1 were used to determine, for each alcohol, the concentration required to give 50% inactivation of the virus. The results (Table 1) show that tetradecanol is effective against ϕ6 by this assay at lower concentrations than any of the other straight-chain alcohols. Butanol, hexadecanol, and octadecanol gave insignificant inactivation at concentrations up to 1 mM, the highest tested. In comparing the three most active alcohols with data previously reported for BHT (15), it may be noted that dodecanol and tetradecanol are active against ϕ6 at lower concentrations than for BHT, whereas about three times more decanol than BHT is required to give 50% inactivation.

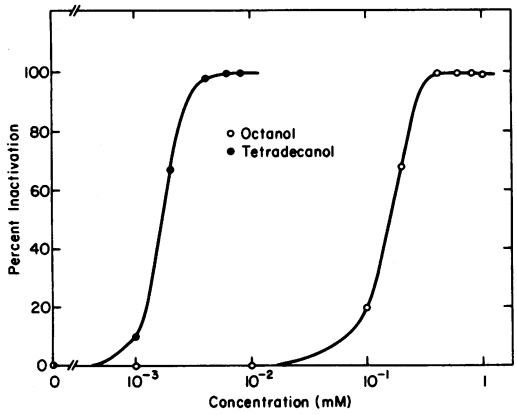

FIG. 1. *Inactivation of ϕ6 by different concentrations of octanol and tetradecanol. The virus was exposed in a Tris-buffered salts solution (see Materials and Methods) for 30 min at room temperature. Initial virus titer at the start of exposure was approximately 10^6 to 2 $\times$ 10^6 PFU/ml.*

TABLE 1. *Concentrations required for 50% inactivation of virus by saturated alcohols of varying chain length*

Alcohol chain length	Conc of alcohol required (mM)	
	ϕ6	ϕ23-1-a
4	>1	>1
6	0.15	>1
8	0.15	>1
10	0.03	>1
12	0.007	>1
14	0.002	>1
16	>1	>1
18	>1	>1

ϕ23-1-a showed no susceptibility to inactivation by any of the alcohols tested. The highest concentration used was 1 mM.

Inactivation of PM2. In general, PM2 is less susceptible to the straight-chain alcohols than ϕ6 but was readily inactivated by several compounds at the higher concentrations. The profile for inactivation as a function of chain length was similar, but not identical, to that for ϕ6. Data taken at a concentration of 0.4 mM are displayed in Fig. 2. For PM2, only decanol and dodecanol inactivate more than 50% of the virus at this concentration. This was also found to be the case in another experiment where a concentration of 1 mM was used. No further experiments were carried out with this virus.

HSV-*syn* 20 and polyoma. The effects of the straight-chain alcohols on two mammalian cell viruses, one that is enveloped and one that contains no lipids, were studied. HSV contains approximately 22% lipid (11) located in a membrane envelope that surrounds the nucleocapsid. HSV-*syn* 20 is a plaque morphology mutant of HSV that causes extensive cell fusion when monolayers are infected in vitro (9). The virions of *syn* mutants of HSV are morphologically indistinguishable from wild-type virus, the dif-

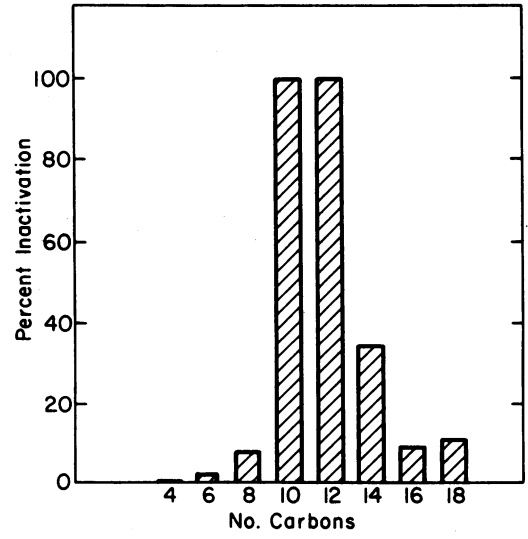

FIG. 2. *Profile for the inactivation of PM2 by saturated alcohols of varying chain length, indicated as the number of carbons on the abscissa. The virus was exposed to 0.4 mM solutions in a Tris-buffered salts solution (see Materials and Methods) with conditions otherwise as stated for Fig. 1.*

ference apparently being due to the patterns of glycosylation of membrane proteins during infection (7). We determined the effects of straight-chain alcohols on HSV-*syn* 20; data for a 30-min exposure to 0.5 mM solutions are shown in Fig. 3. The alcohols that are most active against HSV-*syn* 20, as was the case for ϕ6, are decanol, dodecanol, and tetradecanol. Dodecanol and tetradecanol are also active at lower concentrations (Table 2).

Polyoma is a small deoxyribonucleic acid virus that productively infects mouse cells and transforms hamster cells. It has a diameter of about 45 nm and is non-enveloped. We carried out a limited number of experiments with polyoma and the alcohols phytol and dodecanol. At a concentration of 0.5 mM, polyoma was not inactivated by either compound (data not shown), further substantiating that inactivation by alcohols is due to their effects on virus membranes.

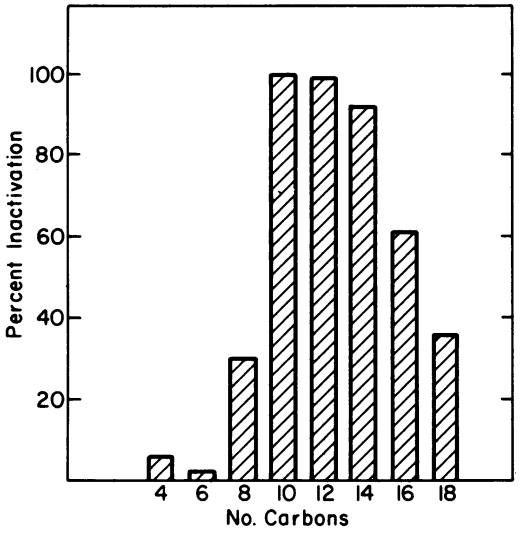

FIG. 3. *Profile for the inactivation of HSV-syn 20 by saturated alcohols of varying chain length. The virus was exposed to 1 mM solutions in a tricine-buffered salts solution (see Materials and Methods) with conditions otherwise as for Fig. 1.*

TABLE 2. *Inactivation of HSV-syn 20 by different concentrations of the most active alcohols*

Alcohol chain length	Conc (mM)	% Inactivation
10	0.5	>99
	0.05	<10
	0.005	<10
12	0.5	>99
	0.05	98
	0.005	<10
14	0.5	91
	0.05	77
	0.005	14

Effects of straight-chain alcohols on host cells. For the bacterial host cells BAL-31 and HB10Y, experiments were carried out to quantify the effects of alcohols on the growth rate of the cells. Cultures growing exponentially were diluted fivefold into medium containing alcohol at 0.5 mM final concentration and further incubated with aeration at 25°C. Growth was monitored by measuring the absorbance at 620 nm using a Bausch and Lomb Spectronic 20 colorimeter. When necessary, corrections were made for the turbidity contributed by the alcohol suspension. A quantitative estimate of growth rate was calculated as the reciprocal of the doubling time for each culture. These data, normalized to that for control cultures, are presented in Table 3. For both BAL-31 and HB10Y, decanol at 0.5 mM caused considerable cell lysis, so no growth rate could be determined. For all the other alcohols the cells grew well, although at reduced rates for octanol and dodecanol. Comparison of these data with those for ϕ6 and PM2 show that the viruses are far more susceptible to dodecanol and tetradecanol than are their host cells.

HEL cells. Monolayers of HEL cells were treated for 2 h with different concentrations of the straight-chain alcohols and then observed for the appearance of any noticeable cytopathic effect. The experiment was performed in such a manner that the samples were scored without knowledge of the conditions of treatment. The results of this experiment are reported in Table 4. At the highest concentration, 0.5 mM, decanol caused cell lysis and both dodecanol and tetradecanol caused observable changes in the monolayer of cells. At 0.05 mM, no cytopathic effect was observed for any of the alcohols. Significant inactivation of HSV-*syn* 20 occurs at 0.05 mM for both dodecanol and tetradecanol,

TABLE 3. *Effect of 0.5 mM saturated alcohols on growth rate of host bacterial cells*

Alcohol chain length	Relative growth rate[a]	
	HB10Y	BAL-31
6	1.0	1.03
8	0.91	0.75
10	Lysed	Lysed
12	0.77	0.43
14	0.93	1.03
Control	1.00	1.00

[a] Calculated as the reciprocal of doubling time for exponentially growing cultures, normalized to the control.

again indicating that the virus is more susceptible than the host cell to these compounds.

Erythrocyte lysis. A more quantitative comparison of the effects of different alcohols on cells of human origin was obtained with erythrocytes. Freshly drawn blood was centrifuged at low speed, and the cells were resuspended in 0.88% NaCl and washed twice in the same saline solution. Samples were treated for 30 min at 37°C with the different alcohols at 0.5 mM final concentration. After removal of unlysed cells by centrifugation and filtering through a 0.22-μm membrane filter (Millipore Corp.), the absorbance of hemoglobin in the filtrate was measured at 540 nm. To express the data as percentage of lysis, a sample was sonicated to cause complete lysis and then processed in the same manner. Figure 4 shows a profile for the effect of the straight-chain alcohols on erythrocytes. Only decanol and dodecanol caused lysis that was significantly greater than untreated controls. These results, along with those for HEL, BAL-31, and HB10Y cultures, clearly indicate that of the alcohols, decanol has the most harsh effect on the cells. Furthermore, these collective data suggest that dodecanol and tetradecanol are the two straight-chain alcohols that show the most promise as potential antiviral agents.

TABLE 4. *Cytopathic effect observed for HEL cells treated for 2 h with different concentrations of alcohols*

Chain length	Conc (mM)	Remarks
4	0.5	No effect
6	0.5	No effect
8	0.5	Few vacuoles
10	0.5	Many cells lysed
	0.05	No effect
12	0.5	About half of the cells off glass; remainder rounded
	0.05	No effect
14	0.5	Few vacuoles
	0.05	No effect
16	0.5	Few off glass; remainder normal
18	0.5	No effect

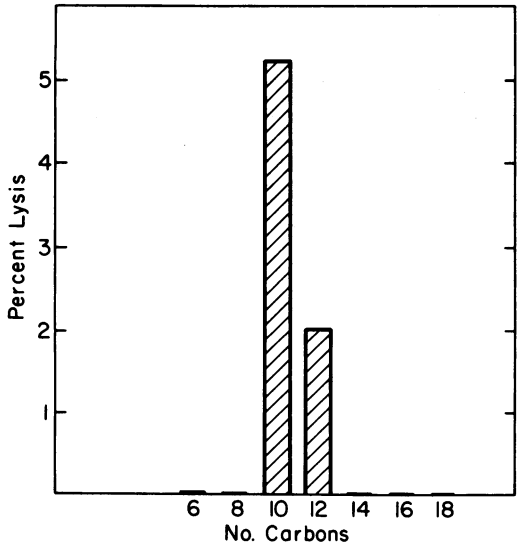

FIG. 4. *Lysis of erythrocytes by saturated alcohols of varying chain length. The cells were exposed to 1 mM solutions in 0.88% NaCl solutions for 30 min at 25°C. Hemoglobin absorbance at 540 nm was measured in the supernatants and compared with that for a control artificially lysed by sonication.*

Phytol. In addition to the experiments just described, we investigated the antiviral potential of phytol, a branched-chain alcohol that occurs in nature as a component of chlorophyll. Its structure is shown in Fig. 5. Data for the effects of phytol on the different viruses are given in Fig. 6 and Table 5. ϕ6 is unusually

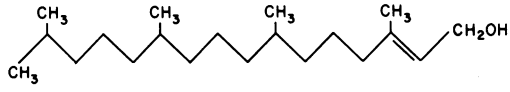

FIG. 5. *Structure of phytol.*

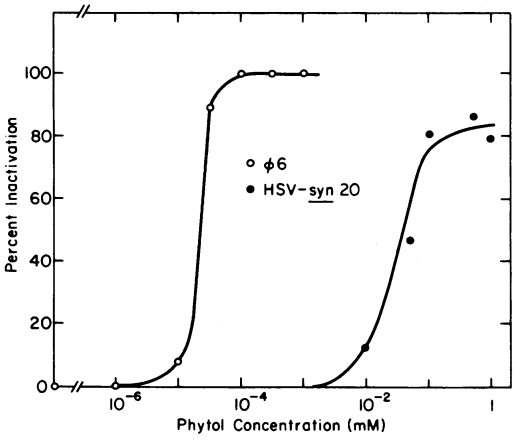

FIG. 6. *Inactivation of ϕ6 and HSV-syn 20 by different concentrations of phytol. Conditions are as described in Materials and Methods and in the legends for Fig. 1 and 3.*

TABLE 5. *Concentrations of phytol required for 50% inactivation of different viruses*

Virus	Conc required (mM)
ϕ6	0.00002
ϕ23-1-a	>1
HSV-*syn* 20	0.035
Polyoma	>1
PM2	>1

susceptible to phytol, HSV-*syn* 20 is inactivated at higher concentrations, and PM2 is not affected by this compound. As expected, ϕ23 and polyoma virus were not susceptible to treatment with phytol.

Phytol gave no reduction in growth rate for BAL-31 and HB10Y cells. HEL cells, when exposed to 0.5 mM phytol for 2 h, contained a few vacuoles but otherwise showed little damage. No effect was observed in HEL cells exposed to 0.05 mM phytol. Due to the abundance of phytol as a natural product, it is expected to have low toxicity to humans and other animals.

DISCUSSION

In considering the design and development of successful antiviral agents, an operational distinction can be made between those compounds that inactivate the virus on contact and those that inhibit the further production of infectious virus particles. For contact inactivation, the agent may be removed after a period of treatment and the virus assayed for infectivity in its absence. For inhibition of virus production, the agent is generally present in the infected culture. In either case, to be useful clinically, the substance must possess antiviral activity at concentrations and under conditions where the organism being treated is comparatively unaffected. The available literature on the selective inhibition of viral functions indicates that the most common approach, by far, has been the search for compounds to inhibit virus production, with very little consideration being given to the contact inactivation of viruses. This may be due, in part, to the fact that many viruses are quite resistant to chemicals that are harmful to cells. Thus, the development of a safe agent that would inactivate all or a large proportion of the pathogenic viruses on contact seems quite unlikely.

As research in antiviral chemotherapy advances, it is becoming apparent that many of the active agents may be effective against only one or a few types of viruses. Nevertheless, the discovery of a drug that would successfully control a single viral disease would constitute a significant advance in the field. The fact that membrane-perturbing antiviral agents, by their very nature, are limited to certain classes of viruses does not appear to detract from their usefulness. Of the viruses that are pathogenic to man and domesticated animals, we estimate that approximately half are encolosed in a lipid-containing membrane structure.

In this report, evidence has been presented that phytol and certain long-chain saturated alcohols are quite effective at inactiviting lipid-containing viruses in vitro. Some of these compounds, notably phytol, dodecanol, and tetradecanol, are active against viruses under conditions that appear to be much less detrimental to the host cells. For the different viruses studied, there exists some degree of specificity with regard to which compounds are most active against which viruses. Phytol, for example, is the most active compound against ϕ6 but is without effect on PM2. Decanol, on the other hand, is more active than tetradecanol against PM2, with the reverse being the case for ϕ6. HSV-*syn-20* appears more susceptible than either ϕ6 or ϕ23 to alcohols having chain lengths greater than 14 carbons. The reasons for and the nature of these specificities are not known at the present time. We anticipate that the structure-function relationships that result in one virus being more susceptible than another to a membrane perturber may also be related to the reasons why a virus may be more susceptible to inactivation than its host cell.

Our findings suggest that dodecanol, tetradecanol, and phytol warrant further studies as potential antiviral agents. It will be of special interest to determine if membrane perturbers that inactivate enveloped viruses on contact do so by inactivating envelope proteins, perturbing lipids, or interrupting lipid-protein interactions. The more thorough our understanding of the similarities and differences between virus and cell membranes, the more rational will be our design and development of this class of antiviral agents.

ACKNOWLEDGMENTS

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